Agilent High Sensitivity Protein 250 Kit Guide

Agilent Technologies

Notices

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Software Revision

This guide is valid for B.02.06 and higher versions of the Agilent 2100 Expert software, where 06 refers to minor revisions of the software that do not affect the technical accuracy of this guide.

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Contents

Agilent High Sensitivity Protein 250 Kit

Table 1 Agilent High Sensitivity Protein 250 Kit (reorder number 5067-1575)

Agilent High Sensitivity Protein 250 Labeling Reagents (reorder number 5067-1577)	
(green)	10x Protein 250 Standard Labeling Buffer (10xSLB), 10-fold concentrate (1 vial)
(clear)	Ethanolamine (1 vial)
(blue)	DMSO (1 vial)
(blue)	Labeling Dye (1 vial, sufficient for three labeling reactions)
High Sensitivity Protein 250 Ladder (reorder number 5067-1578)	
(yellow)	Ladder (1 vial, sufficient for three labeling reactions)
High Sensitivity Protein 250 Reagents (for separation, reorder number 5067-1576) & Supplies	
(red)	Gel-Matrix (1 vial, prefiltered)
(purple)	Destaining Solution (1 vial)
(white)	Sample Buffer (3 vials)
Syringe Kit	
1 Syringe	
High Sensitivity Protein Chips	
10 Chips	
1 Electrode Cleaner	

Table 2 Prerequisites for labeling procedure

Prerequisite	Specification
Type	Lysates, Extracts, Column Fractions, purified Proteins, lyophilized Proteins
Concentration	1 ng/µl to 3 µg/µl total protein
Volume	5 µl per labeling reaction
pH Value	Adjustment to pH 8.0 - 9.0
Optimal Matrix	Standard labeling buffer, supplied
Interferences	All components with primary amino or thiol groups

Table 3 Specifications for analysis of labeled proteins

Type	Specification
Sizing range	10-250 kDa
Typical sizing resolution	10%, based on ladder
Typical sizing accuracy	10% CV (BSA)
Sizing reproducibility	3% CV (BSA)
Quantitative range	up to 4 orders of magnitude (0.3 to 3000 ng/µl BSA)
Sensitivity	1 pg/µl labeled BSA in water on chip with labeling reaction performed at 1 ng/µl total Protein; 5 pg/µl for labeled BSA in PBS
Quantitation reproducibility	20% CV BSA, for sample concentration greater 1 ng/µl labeled protein
Compatible Samples	Diluted, fluorescently labeled proteins (See "Labeling Reaction with Fluorescent Dye" on page 24*.)

BSA = Bovine Serum Albumin

* This document is also available through the Help-menu of the 2100 Expert Software under "related documents". For latest revisions, visit Agilent Technologies' web site on high sensitivity *http://www.agilent.com/chem/2100-protein250*.

Table 4 Physical Specifications

Type	Specifications
Analysis run time	30 minutes
Number of samples	10 samples/chip
Sample volume	4 µl labeled protein on chip
Kit stability	6 months at -20 °C

Required Equipment for Agilent High Sensitivity Protein 250 Assay

Equipment Supplied with the Agilent 2100 Bioanalyzer

- Chip priming station (reorder number 5065-4401)

Additional Material Required (Not Supplied)

- 0.5 ml tubes*
- Vortexer
- Deionized water
- Microcentrifuge
- 0.5 ml heating block or water bath (95-100°C)
- pH-meter or indicator strips (basic range)

Check the Agilent Lab-on-a-Chip webpage for details on assays: www.agilent.com/chem/labonachip.

Agilent High Sensity Protein 250 Assay Principles

The complete Agilent High Sensitivity Protein 250 kit contains chips and reagents for labeling of proteins with a dedicated fluorescent dye and subsequent sizing and quantitation.

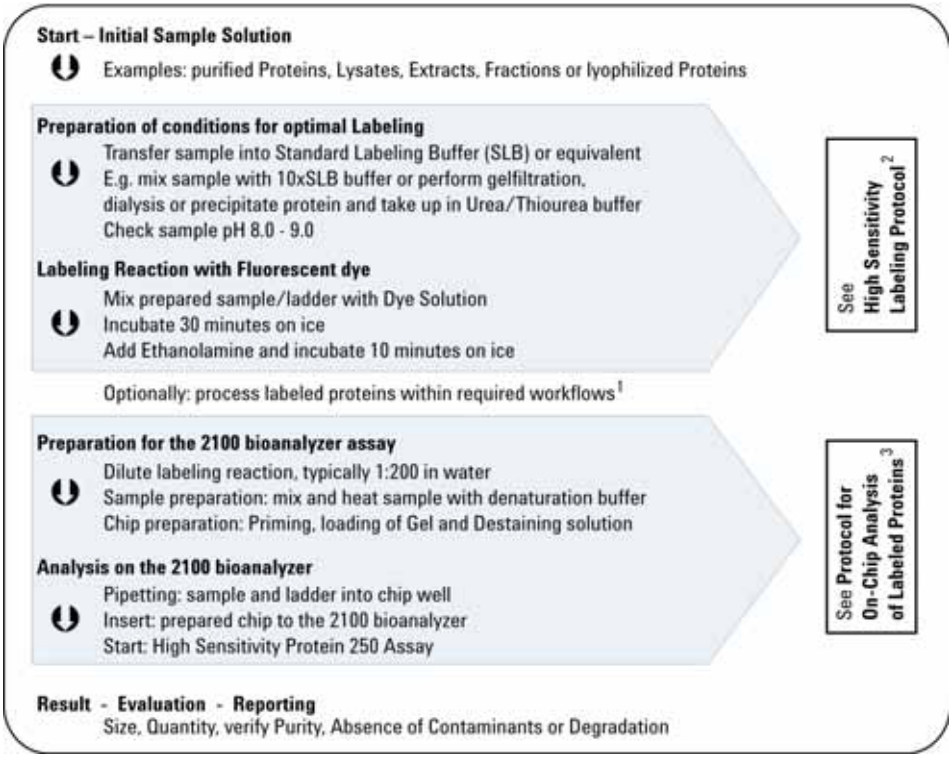
The Assay workflow consists of two major steps:

- Covalent labeling of proteins with a fluorescent dye
- Separation and detection of labeled proteins with on-Chip-Electrophoresis

Epsilon-amino groups of lysine residues of proteins are covalently modified with a fluorescent dye by N-Hydroxy succinimidyl (NHS) ester chemistry.

Prepared 2100 bioanalyzer chips consist of an interconnected set of polymer filled microchannels that sieve proteins by size as they are driven through it by means of electrophoresis. Agilent Protein kits are designed for use with the Agilent 2100 bioanalyzer only.

General workflow for the High Sensitivity Assay



[1] Alternative workflows may be any purification, depletion or fractionation technique (e.g. by 3100 OFFGEL fractionator, Agilent Technologies). See "Alternative Workflows" on page 29

[2] See "High Sensitivity Labeling Protocol" on page 17

[3] See "Protocol for On-Chip Analysis of Labeled Proteins" on page 27

Setting up Assay Equipment and Bioanalyzer

Before beginning the labeling procedure:

You have to ensure that

- the sample is prepared for optimal labeling conditions (see "Preparation of Optimal Conditions for Labeling" on page 17).
- the labeling dye is reconstituted (see "Reconstitution of Fluorescent Dye with DMSO" on page 25).
- an ice bath is available

Before beginning the chip preparation protocol, ensure that the chip priming station and the bioanalyzer are set up and are ready to use.

You have to

- replace the syringe at the chip priming station with each new protein kit
- adjust the base plate of the chip priming station to position A
- adjust the syringe clip at the chip priming station to the middle position
- adjust the bioanalyzer's chip selector to position 1
- ensure that a heating block (95-100°C) is available for heat denaturation
- start the 2100 Expert software (Revision B.02.06 or higher), connect successfully to a 2100 bioanalyzer and load the High Sensitivity Protein 250 assay before you load a chip.

NOTE The Agilent High Sensitivity Protein 250 assay is an assay which requires staining of proteins with a dedicated labeling protocol. The labeling is done prior to the analysis on chip. Please read this guide carefully and follow all instructions to guarantee satisfactory results.

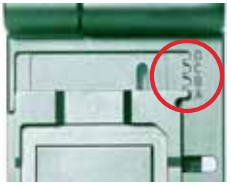
Setting up the Chip Priming Station

NOTE Replace the syringe with each new Reagent Kit.

1

- **a** Unscrew the old syringe from the lid of the Chip Priming Station.
- **b** Release the old syringe from the clip. Discard the old syringe.
- **c** Remove the plastic cap of the new syringe and insert it into the clip.
- **d** Slide it into the hole of the luer lock adapter and screw it tightly to the Chip Priming Station.

2 Adjust the base-plate:



- **a** Open the chip priming station by pulling the latch.
- **b** Using a screwdriver, open the screw at the underside of the base plate.
- **c** Lift the base plate and insert it again in position A. Retighten the screw.

3 Adjust the syringe clip:

- **a** Release the lever of the clip and lift it up or down to adjust it to the middle position.

Setting up the Bioanalyzer

Adjust the chip selector:

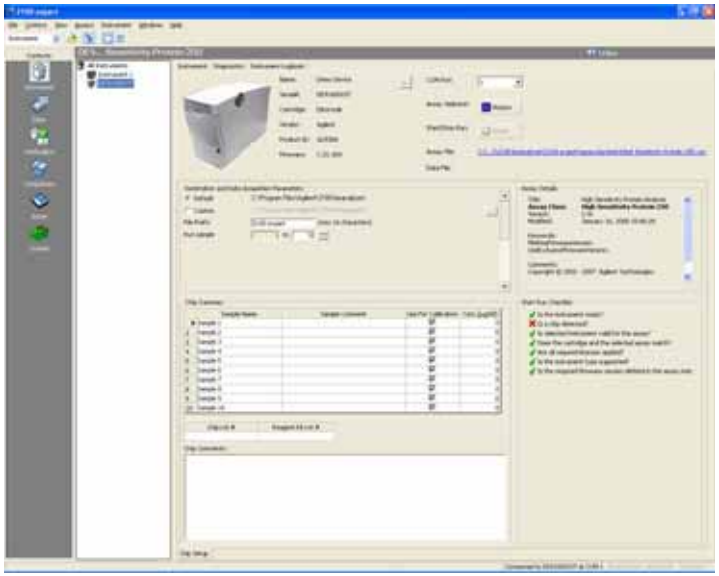
1. Open the lid of the bioanalyzer and make sure that the electrode cartridge is inserted in the instrument. If not, open the latch, remove the pressure cartridge and insert the electrode cartridge.
2. Remove any remaining chip and adjust the chip selector to position (1).

Starting the 2100 Expert Software

To start the software:

1. Go to your desktop and double-click the following icon.

The screen of the software appears in the*Instrument* context. The icon in the upper part of the screen represents the current instrument/PC communication status:



Lid closed, no chip or chip empty

Lid open

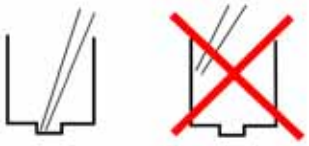
Dimmed icon: no communication

Lid closed, chip inserted

2 If more than one instrument is connected to your PC, select the instrument you want to use in the tree view.

Essential Measurement Practices

- Handle and store all reagents according to the instructions under storage conditions.
- Avoid sources of dust or other contaminants. Foreign matter in reagents and samples or in the wells of the chip will interfere with assay results.
- Allow all reagents and samples to equilibrate to room temperature for 30 minutes before use and vortex.
- Always insert the pipette tip to the bottom of the chip well when dispensing the liquid. Placing the pipette at the edge of the well may lead to poor results.



- Use a new syringe and electrode cleaners with each new kit.
- Use loaded chips within 5 minutes. Reagents might evaporate, leading to poor results.
- Do not touch the Agilent 2100 bioanalyzer during analysis and never place it on a vibrating surface.
- Use 0.5 ml tubes to denature samples. Using larger tubes may lead to poor results, caused by evaporation.
- Keep suitable aliquots of the labeling reaction of the High Sensitivity Protein 250 Ladder undiluted at -20°C. Avoid freeze thaw cycles to prevent precipitation.
- The High Sensitivity Protein 250 Assay Gel-Matrix comes pre-filtered. It is ready to use after thawing.
- For Protein analysis under reducing conditions a 1 M DTT solution is required.
- Samples from labeling reactions need to be diluted prior to analysis. Do not dilute heat denatured samples.

- Relative concentrations indicated in the Peak Table of the Expert Software may need correction for the dilution step (e.g. 1:200). See "Checking your Agilent High Sensitivity Protein 250 Results" on page 38.
- Use calibrated pipets with proper pipetting technique. Choose suitable pipets and tips for low volumes.

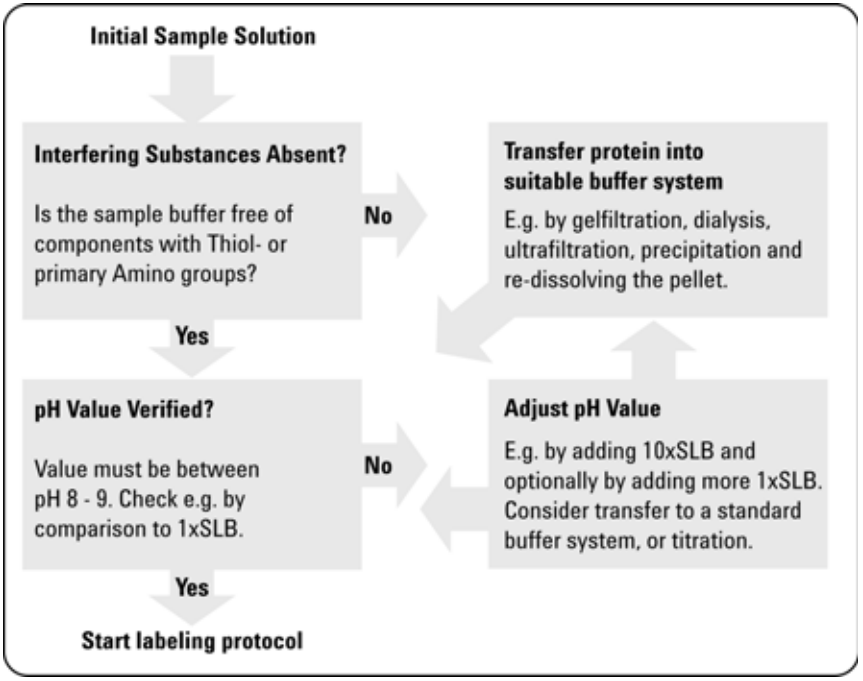
Storage Conditions:

- Keep all reagents frozen at -20°C when not in use to avoid poor results caused by reagent decomposition.
- Avoid freeze thaw cycles for the ladder preparation.
- Protect all following reagents from light: Labeling Dye, Sample Buffer, Destaining Solution and any Dye-labeled ladder/protein solution.
- Remove light covers only when pipetting. Dye decomposes when exposed to light.
- Store chips at room temperature.

High Sensitivity Labeling Protocol

Preparation of Optimal Conditions for Labeling

Follow all instructions for adjusting optimal labeling conditions when preparing the samples. It is crucial to have sample solutions in the pH range from pH 8-9 for satisfactory results of the complete assay. Note that it is essential as well to avoid interfering substances (see "Compatibility List for the Labeling Reaction" on page 45) in sample solutions.



Verification of Absence of Interfering Substances

The solution with the sample protein should not contain disturbing components with Thiol- or primary Amino groups. Negative Interference on the labeling efficiency is known from primary amine or thiol groups of buffer components such as DTT, ß-Mercaptoethanol, Glutahione, free Aminoacids and Imidazole. These buffer components compete with the sample protein in the reaction for the reactive labeling reagent (fluorescent dye). This competition lowers the effective reagent concentration and the amount of dye bound to protein, which subsequently lowers peak areas.

Interference from detergents such as CHAPS, Triton X-100, SDS or Tween 20 may have an influence the overall assay performance. Although labeling efficiency may be enhanced the analysis of labeled protein is affected in sizing and quantitation with on-Chip-Electrophoresis at higher detergent concentrations.

It is recommended to test any buffer deviating from the standard labeling buffer on its suitability for labeling by directly comparing it to a recommended buffer (see "Buffer Systems Suitable for the Labeling Reaction" on page 23).

NOTE It was found that a concentration of 1 mM DTT present in the Standard Labeling Buffer reduced the peak area of dedicated immunoglobulines by half. DTT contains thiol groups.

NOTE Find a comprehensive list of compatible sample buffers and possible effects on the labeling reaction under "Compatibility List for the Labeling Reaction" on page 45.

Verification of Sample pH

Optimal labeling with the fluorescent dye will take place only if the pH is between pH 8.0 and pH 9.0. It is crucial to actively check the pH of sample solutions prior to the labeling reaction.

Verification of the pH value for the labeling reaction can be done by transferring a droplet to a pH-indicator strip which focuses on the basic range. Alternatively use a pH-meter on aliquots of your sample or buffer.

Labeling with the fluorescent dye will take place only if the pH is greater than pH 8.0. In pH-ranges beyond pH 9.0 other amino acid groups will be labeled as well. Both deviations from the optimum will negatively influence the assay results. Uniform labeling efficiency across all samples and the ladder is required for good quantitation results and reproducibility.

NOTE For control of pH values special indicator strips focused on pH range 6.5 - 10.0 (0.2 - 0.5 unit resolution, Merck KGaA Darmstadt) were used successfully.

NOTE Consider the usage of micro pH electrodes for measurements of small sample amounts.

NOTE Labeling efficiency is greatly influenced by the pH value of the sample matrix.

pH Adjustment for Samples

In case the sample shows a pH below pH 8.0 or above pH 9.0 it is necessary to adjust it to around pH 8.5 prior to the labeling reaction.

Dilution of sample with 10xSLB:

Standard labeling buffer is supplied as 10-fold concentrate (10xSLB, ●). To shift the pH add one part 10xSLB to 9 parts of sample. Consider this dilution factor in quantitation later on. Make sure the buffer capacity is suitable to shift the pH into a pH range between pH 8.0 and pH 9.0. Starting from the 10x concentrate the 1xSLB final concentration is 30 mM Tris. An estimation for

the buffer capacities can be done from the molarities of the buffering substance in your individual sample buffer. The molarity of initial sample buffer substances should be much lower than 30 mM.

Confirm the adjusted pH prior to the labeling reaction by re-testing the solution. A good comparison for color pH-indicator strips is to test an aliquot of 1xSLB in parallel.

In case the pH adjustment with the above given recommendation is not applicable to your sample matrix consider to transfer sample proteins into a suitable buffer (see "Buffer Systems Suitable for the Labeling Reaction" on page 23).

NOTE For example PBS (26mM NaH_2PO_4; 41mM Na_2HPO_4, 79mM NaCl, pH 7.4) as sample matrix can be shifted in pH adequately by the addition of 1/10 volume 10xSLB to a pH > 8 which is suitable for labeling.

NOTE Optionally more 1xSLB can be used to shift the pH furthermore. Do not exceed a final Tris concentration higher than 100 mM. Tris contains an amino group that may influence the labeling reaction at higher concentrations.

NOTE In case addition of 10xSLB is not sufficient to shift the pH to the desired range, consider careful addition of 50mM NaOH. Titrate your sample matrix offline to determine the required amount. Make sure the proteins under investigation are not harmed by this procedure.

NOTE Deionized water should be at a neutral pH. Please check prior to use.

Transfer of Protein into Suitable Buffer

In case the sample is in a buffer matrix that contains interfering substances it is recommended to transfer the proteins of interest to a suitable buffer, prior to the labeling reaction. Such suitable buffer may be the 1-fold concentrated standard labeling buffer (1xSLB), the recommended Sodiumbicarbonate buffer or the recommended Urea/Thiourea buffer (see "Buffer Systems Suitable for the Labeling Reaction" on page 23). Any other deviating buffer should be tested in direct comparison for equivalent efficiency in labeling.

Generally the following methods for transfer can be used:

Gelfiltration

Gelfiltration e.g. in spin cartridges can be used for convenient removal of small sized interfering substances and buffer exchange. Equilibrate the resin with 1xSLB, Sodiumcarbonate or Urea/Thiourea buffer and proceed according to the manufacturers instructions. If buffer is exchanged completely to a recommended buffer, further pH adjustment is not necesary. Please verify the sample pH. Consider the final sample volume and dilution factors in quantitation.

NOTE

The Zeba™ Micro Desalt Spin Columns (Pierce) were successfully used to transfer unknown samples into Standard Labeling Buffer or Sodiumbicarbonate buffer for the labeling reaction.

Dialysis

Dialysis should be done with equilibrated membranes featuring suitable size cut-offs (size-retention e.g. at 3 kDa or 10 kDa) in accordance with the expected protein size. Dialysis should be performed for a sufficient time and with adequate buffer exchanges. If buffer is exchanged completely to a recommended buffer, further pH adjustment is not necesary. Please verify the sample pH.

Ultrafiltration

Ultrafiltration e.g. in spin cartridges can be used for convenient re-concentration after a sample was diluted e.g. with 10x or 1x standard labeling buffer. Small interfering substance can be reduced in concentration by this procedure. The membrane should be equilibrated with e.g. 1xSLB and the cut-off (size-retention e.g. at 3 kDa or 10 kDa) should be in accordance with protein size.

Dissolving a pellet of sample proteins

This method requires precipitation and solubilization of sample proteins. Impurities are depleted effectively unless they are co-precipitated. Solubilization into the recommended Urea/Thiourea buffer (see "Buffer Systems Suitable for the Labeling Reaction" on page 23) can be done in small scale and enables concentration of the initial sample content. A simple acetone precipitation protocol is given below.

Simple clean-up protocol for proteins by acetone precipitation:

- Add 4-fold volume of chilled Acetone (-20°C, not supplied)
- Incubate for ≥ 30 minutes at -20°C
- Pellet the protein by centrifugation: 10 minutes, 15,000g, 4°C
- Optionally: Wash pellet with a chilled 4:1-mixture of Acetone:Water
- Remove supernatant completely and let pellet dry at room temperature
- Add Urea/Thiourea buffer to the pellet and dissolve to e.g. 1 µg/µl

Dissolving a lyophilisate

Take up a solid protein lyophilisate free of interfering substance into a suitable buffer e.g. 1xSLB. Lyophilization prerequisite is that the sample protein is dissolved in a completely volatile buffer. Verify absence of interfering substance in the lyophilisate. Note that not all proteins are stable during the freeze-drying process.

NOTE The 2-D Clean-Up kit (GE Healthcare) was successfully used to prepare unknown samples for the labeling reaction.

Buffer Systems Suitable for the Labeling Reaction

Standard labeling buffer (SLB): 30 mM Tris/HCl, pH 8.5

The Standard labeling buffer is supplied with the kit as 10-fold concentrate (10xSLB; 300 mM Tris/HCl, pH > 8.5). It can be added to samples in order to shift the pH into the suitable range. To obtain a 1xSLB e.g. for buffer exchange in gel filtration or for comparison in pH-measurements, dilute the 10xSLB 1:10 with deionized water. E.g. add 10µl 10xSLB to 90µl water.

Sodiumbicarbonate buffer: 100 mM $NaHCO_3$

Preparation of 50 ml: accurately weigh in solid Sodiumbicarbonate ($NaHCO_3$, 420 mg), add water, stir until complete dissolution and fill up to 50 ml. Sodiumbicarbonate buffer decomposes with time. Prepare fresh frequently, store e.g. 2 weeks at 4°C at the most. Check pH value to be between pH 8.0 and 9.0 prior to use.

Urea/Thiourea buffer: 30 mM Tris/HCl, 7 M Urea, 2 M Thiourea, pH 8.5

Preparation of 500 µl: accurately weigh in solid Urea (210 mg) and Thiourea (76 mg), add 50 µl of 10xSLB and add 250 µl deionized water. Mix until complete dissolution of solid components and fill up with deionized water to 500 µl. Urea/Thiourea buffer decomposes with time. Prepare fresh frequently, store e.g. 2 month at -20°C at the most. Check pH value to be between pH 8.0 and 9.0 prior to use.

NOTE Deionized water should be at a neutral pH. Please check prior to use.

NOTE Ammonium carbonate buffers are not suitable for the labeling reaction since the Ammonium competes with the protein for the reactive Fluorescent Dye and thus interferes with the labeling reactions.

Labeling Reaction with Fluorescent Dye

WARNING

Handling Reagents

Wear hand and eye protection and follow good laboratory practices when preparing and handling reagents and samples. For kit components that are hazardous the following risk and safety phrases (Europe) apply.

- ➔ Fluorescent Dye is Irritant (Xi) please regard: R: 41 and S: 22, 26, 39.
- ➔ Ethanolamine is Corrosive (C) please regard R: 20/21/22, 34 and S: 1/2, 26, 36/37/39, 45.
- ➔ Lithium dodecyl sulfate (LiDS) in solution is Harmful (Xi), please regard R: 36/37/38 and S: 26-36.
- ➔ For Methylurea no hazard code applies, please regard R: 22 and S: 22-36.
- ➔ Please regard the Materail Safety Data Sheet for this kit. It is available under www.agilent.com.

Other kit components, namely DMSO, have no code for hazardous potential or associated risk and safety phrases. Please handle the DMSO stock solutions with particular caution as DMSO is known to facilitate the entry of organic molecules, such as the fluorescent dye, into tissues.

Epsilon-amino groups of lysine residues of proteins are covalently modified with a fluorescent dye by N-Hydroxy succinimidyl (NHS) ester chemistry.

The Fluorescent dye reagent needs reconstitution in DMSO. For the labeling reaction it is mixed with sample solution or ladder. Excess dye will be quenched after the reaction by Ethanolamine. Ladder volume provided in this kit is sufficient for 3 independent labeling reactions. Each Ladder labeling reaction may be aliquoted and used successively for several chips.

Reconstitution of Fluorescent Dye with DMSO

1. Remove the fluorescent dye and DMSO from the freezer (●, both vials).
2. Equilibrate both vials to room temperature, DMSO should be completely thawed.
3. Centrifuge dye vial at 10,000g for 2 min to collect solid dye particles at the bottom of the vial.
4. Visually localize the dye pellet and add 54 µl DMSO onto the pellet.
5. Vigorously vortex the Dye solution until all solid components are completely dissolved.
6. Label the vial with date.
7. Store Dye solution in the dark at -20°C for up to 6 months, thaw completely prior to use.

NOTE DMSO is solid at 4°C. Please thaw DMSO and dye solution completely. Vortex the reconstituted dye prior to use.

Performing the Labeling Reaction

1. Thaw reconstituted Dye solution completely and vortex prior to use.
2. Prepare 5 µl of Ladder (●) in a tube.
3. Prepare 5 µl of protein sample (ready for labeling, see "Preparation of Optimal Conditions for Labeling" on page 17) per tube.
4. Place tubes on ice.
5. Start the labeling reaction by adding 0.5 µl of reconstituted Dye solution (●) to tubes with samples or ladder, vortex and spin down for 5 seconds.
6. Incubate 30 minutes on ice.
7. In order to quench any excess of dye add 0.5 µl of Ethanolamine (▢), vortex and spin down for 5 seconds.
8. Incubate 10 minutes on ice.

   → Labeling of Ladder and protein sample is finalized.

9 Start analysis of the labeled Ladder and store remaining solution aliquoted (1 µl) at -20°C.

10 Start analysis of the labeled products or store labeling reaction mixture at -20°C.

NOTE Performing the reaction on ice ensures uniform and reproducible protein labeling.

NOTE Consider the use of tubes with low binding capacity for work with minimal amounts of protein. Eppendorf Safe-Lock Protein LoBind 0.5 ml Microcentrifuge tubes were tested successfully for the labeling reaction, dilution steps and storage.

Protocol for On-Chip Analysis of Labeled Proteins

Protocol for On-Chip Analysis of Labeled Proteins

The on-chip analysis of proteins labeled with Fluorescent Dye requires dilution of the initial labeling reaction and heat denaturation prior to sample loading to the prepared chip.

1. After completing the initial steps in "Setting up Assay Equipment and Bioanalyzer" on page 11, you can perform the steps, as described in the following procedures.
2. Allow the Protein 250 gel matrix and destaining solution to equilibrate to room temperature for 30 minutes. Both solutions need no further preparation prior to use.
3. Allow the High Sensitivity Protein 250 denaturing solution to equilibrate to room temperature for 30 minutes. Prior to the first usage a preparation is needed, as described below.

NOTE All kit components can be stored at -20°C.

NOTE It is important that all the reagents have room temperature before starting the next steps.

NOTE Protect the dye containing solutions such as labeled samples, sample buffer, denaturing solution and destaining solution from light.

NOTE An entry of sample names and comments (e.g. dilution factor) is possible in the instrument context prior to starting a run.

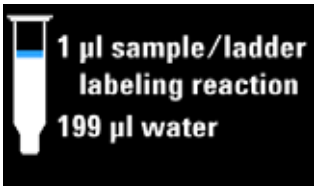
Dilution of Labeled Proteins

For analysis of samples directly from the labeling reaction:

1 Allow labeling reaction mix from the samples to equilibrate to room temperature.

2 Allow an aliquot from the ladder labeling reaction to equilibrate to room temperature.

3 Dilute sample and ladder 1:200 in water, e.g. add 1 µl of the labeling reaction to 199 µl water, vortex, and continue immediately with the on-chip analysis.



NOTE In case a different dilution factor is considered in your customized workflow, please regard potential consequences (see "High Sensitivity Protein 250 Sample Well Results" on page 39). For alternative dilution buffers see "List of Known Effects from Dilution Buffers" on page 47.

NOTE Dilution is necessary to avoid signal saturation and subsequent bias. Often, this is due to the Lower Marker peak, representing co-migrating excess dye from the labeling reaction and Lower Marker from the sample buffer.

NOTE Diluted labeled sample and ladder should be analyzed immediately. Do not use this preparation after storage since proteins may precipitate, degrade or aggregate.

Alternative Workflows

In case of proceeding alternative workflows with the labeled sample proteins the dilution step can be adapted. An alternative workflow may be any preparative technique. Labeling of lysates, extracts or other mixtures of proteins can also be done prior to their separation. Initially labeled proteins can serve as starting material in size exclusion, hydrophobic interaction, ion exchange chromatography, precipitation or separation according to isoelectric points (e.g. by Agilent 3100 OFFGEL fractionator). The multiple fractions of these approaches can subsequently be analyzed directly with the High Sensitivity Protein 250 Assay.

The described workflows usually implicate a depletion of the non-reacted dye. Therefore no or only little dilution is necessary. E.g. 3100 OFFGEL fractionations of labeled proteins can be analyzed without further dilution.

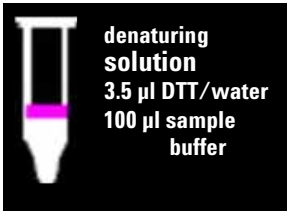
Preparing the Denaturing Solution

1 Remove one of the original vials of the High Sensitivity Protein 250 sample buffer (white ○) or a vial with an aliquot of the High Sensitivity Protein 250 sample buffer from the freezer. Allow to equilibrate to room temperature for 30 minutes, then vortex.

2 For *reducing* conditions: To the 100 µl sample buffer in the original vial (white ○) add 3.5 µl of 1 M Dithiothreitol (DTT) solution or 3.5 Vol.-% of 1 M DTT to an aliquot of sample buffer.

- OR -

For *non-reducing* conditions: Add deionized water to sample buffer instead of DTT.



3 Vortex for 5 seconds.

NOTE The denaturing solution contains a Fluorescent Dye functioning as lower marker in the assay. Protect this solution from light.

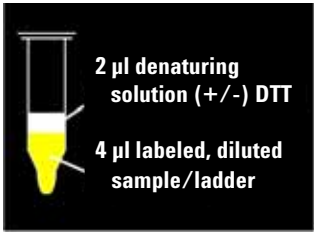
NOTE 100 µl denaturing solution is sufficient for 50 sample preparations. Store prepared denaturing solution at -20°C.

NOTE Avoid exposure of DTT containing buffer to the air since oxidation may degrade the reducing reagent leading to insufficient reduction of your sample proteins.

Preparing the Samples and the Ladder

For a list of compatible buffers for the dilution step or any matrix remaining from alternative workflows, please refer to "List of Known Effects from Dilution Buffers" on page 47.

1. Allow the denaturing solution (prepared as described above) to equilibrate to room temperature for 30 min, and vortex before use.
2. Combine 4 µl of your labeled, diluted protein sample and 2 µl of denaturing solution in a 0.5 ml microcentrifuge tube. Prepare multiples of this for replicates.
3. Combine 4 µl of your labeled, diluted High Sensitivity Protein 250 Ladder and 2 µl of denaturing solution in a 0.5 ml microcentrifuge tube.



4. Mix well and spin down for 15 seconds.
5. Place each sample tube and the ladder tube for 5 minutes in a heating block at 95-100°C or in boiling water.

   Ensure that the tubes are properly placed and heated. Do not heat for more than 5 minutes otherwise excessive evaporation might occur. The samples and ladder should not dry down.
6. Let the tubes cool down to ambient temperature.
7. Spin vials for 15 seconds to recover condensate or liquid.

   Samples and Ladder are now ready for chip loading.

NOTE You will need 6 µl sample preparation per chip well. No further dilution step is done. Prepare duplicates of samples or ladder in order to fill all 10 wells of a complete chip.

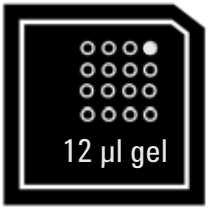
NOTE Ladder performance is uniform for reducing and non-reducing conditions.

NOTE Incubation time and temperature for heat denaturation may require optimization for your protein of interest.

Loading the Gel Matrix

NOTE Before loading the gel matrix, make sure that the base plate of the chip priming station is in position (A) and the adjustable clip is set to the middle position. Refer to "Setting up the Chip Priming Station" on page 12 for details.

1. Allow the gel matrix to equilibrate to room temperature for 30 minutes before use. The gel comes prefiltered and is ready to use after equilibration to room temperature.
2. Take a High Sensitivity Protein chip out of its sealed bag and place it on the Chip Priming Station.
3. Pipette 12 µl of the gel at the bottom of the well marked (◎).

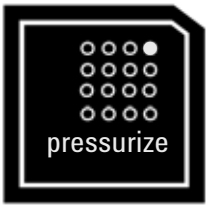


NOTE When pipetting the gel matrix insert the tip of the pipette to the bottom of the chip well when dispensing. This prevents a large air bubble forming under the gel. Placing the pipette at the edge of the well may lead to poor results.

4 Set the timer to 90 seconds, make sure that the plunger is positioned at 1 ml and then close the Chip Priming Station. The lock of the latch will click when the priming station is closed correctly.

5 Press the plunger of the syringe down until it is held by the clip.

6 Wait for exactly 90 seconds and then release the plunger with the clip release mechanism.



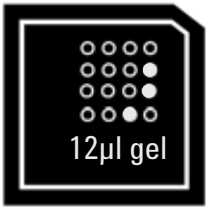
NOTE Use a new syringe with each new chip box. Mount syringe to the chip priming station and test this with the “seal test”. See details in the 2100 Expert Help Menu.

7 Visually inspect that the plunger moves back at least to the 0.3 ml mark.

8 Wait for 5 seconds, then slowly pull back the plunger to the 1 ml position.

9 Open the Chip Priming Station.

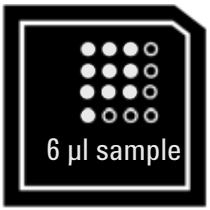
10 Pipette 12 µl of the gel mix in each of the 3 wells marked with G.



11 Pipette 12 µl of the destaining solution in the well marked DS.

Loading the Samples

1 Pipette the complete volume of the heat denatured samples (6 µl, prepared as described in "Preparing the Samples and the Ladder" on page 30) into the sample wells marked 1...10.

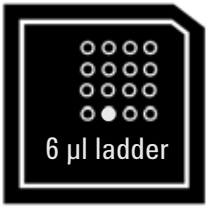


NOTE Complete volume from heat denaturation is potentially less than 6 µl due to evaporation or pipetting inaccuracy. Use tight vials and calibrated pipets.

NOTE In case insufficient liquid levels are filled to the chip wells the 2100 Expert Software will not start the chip run but give a warning. A loss of 5% volume is acceptable.

2 Pipette the complete volume (6 µl) of the heat denatured ladder (as described in "Preparing the Samples and the Ladder" on page 30) into the well marked with the ladder symbol .

3 Make sure that the run is started within 5 minutes. Select the High Sensitivity Protein 250 assay within the instrument context. Refer to the next topic on how to insert the chip in the Agilent 2100 bioanalyzer.



NOTE Do not leave any wells empty or the chip will not run. Pipette a sample or ladder replicate in any empty sample well.

Inserting a Chip in the Agilent 2100 Bioanalyzer

1 Open the lid of the Agilent 2100 bioanalyzer.

2 Check that the electrode cartridge is inserted properly and the chip selector is in position (1). Refer to "Setting up the Bioanalyzer" on page 13 for details.

3 Place the chip carefully into the receptacle. The chip fits only one way.

CAUTION

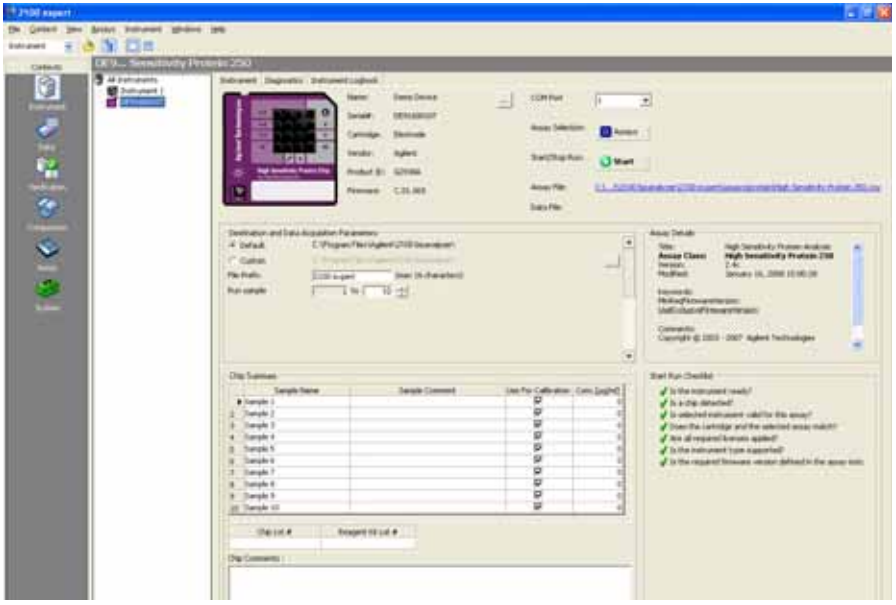
Sensitive electrodes and liquid spills

Forced closing of the lid may damage the electrodes and dropping the lid may cause liquid spills resulting in bad results.

➔ Do not use force to close the lid and do not drop the lid onto the inserted chip.

4 Carefully close the lid. The electrodes in the cartridge fit into the wells of the chip.

5 The 2100 expert software screen shows that you have inserted a chip and closed the lid by displaying the chip icon at the top left of the *Instrument* context.



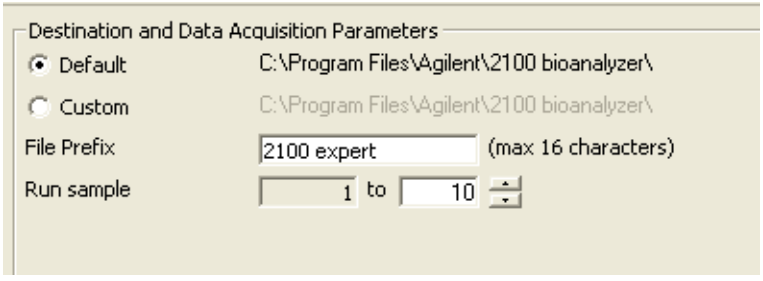
Starting the Chip Run

NOTE Please note that the order of executing the chip run may change if the Agilent Security Pack software (only applicable for Agilent 2100 expert software Revision B.02.06 and higher) is installed. For more details please read the 'User's Guide' which is part of the Online Help of your 2100 expert software.

1 In the **Instrument** context, select the High Sensitivity Protein 250 assay from the Assay menu.

2 Accept the current **File Prefix** or modify it.

Data will be saved automatically to a file with a name using the prefix you have just entered. At this time you can also customize the file storage location and the number of samples that will be analyzed.



3 Click the **Start** button in the upper right of the window to start the chip run. The incoming raw signals are displayed in the **Instrument** context.

4 To enter sample information like sample names and comments, select the *Data File* link that is highlighted in blue or go to the **Data** context and select the **Chip Summary** tab. Complete the sample name table.

NOTE An entry of sample names and comments is also possible in the instrument context prior to starting a run. The information (e.g. dilution factor as comment) can be entered in the chip summary tab while the analysis takes place as well or after the run is finished.

NOTE For aspects of absolute quantitation please refer to "Absolute quantitation" on page 42. For information on adjustment of the ladder concentration refer to "Adjustment of the ladder concentration" on page 43.

5 To review the raw signal trace, return to the *Instrument* context.

CAUTION Contamination of electrodes

Leaving the chip for a period longer than 1 hour (e.g. over night) in the bioanalyzer may cause contamination of the electrodes.

➔ Immediately remove the chip after a run.

6 After the chip run is finished, remove the chip from the receptacle of the bioanalyzer and dispose it according to good laboratory practices.

Cleaning Electrodes after a Chip Run

When the assay is complete, *immediately* remove the used chip from the Agilent 2100 bioanalyzer and dispose it according to good laboratory practice. Then perform the following procedure to ensure that the electrodes are clean (no residues are left over from the previous assay).

NOTE Use a new electrode cleaner with each new kit.

CAUTION

Leak currents between electrodes

Liquid spill might cause leak currents between the electrodes.

➔ Never fill too much water in the electrode cleaner.

1 Slowly fill one of the wells of the electrode cleaner with 350 µl deionized analysis-grade water.
2 Open the lid and place the electrode cleaner in the Agilent 2100 bioanalyzer.
3 Close the lid and leave it closed for about 10 seconds.
4 Open the lid and remove the electrode cleaner.
5 Wait another 10 seconds to allow the water on the electrodes to evaporate before closing the lid.

NOTE

After each chip run, empty and refill the electrode cleaner.

After 10 chip runs, replace the used electrode cleaner by a new one.

NOTE

When switching between different assays, a more thorough cleaning may be required. Refer to the maintenance chapter and Troubleshooting Guide for details. It is part of the Online Help of the 2100 bioanalyzer software.

Checking your Agilent High Sensitivity Protein 250 Results

High Sensitivity Protein 250 Ladder Well Results

To check the results of your run, select the Gel or Electropherogram tab in the Data context. The electropherogram of the ladder well window should resemble the one shown below.

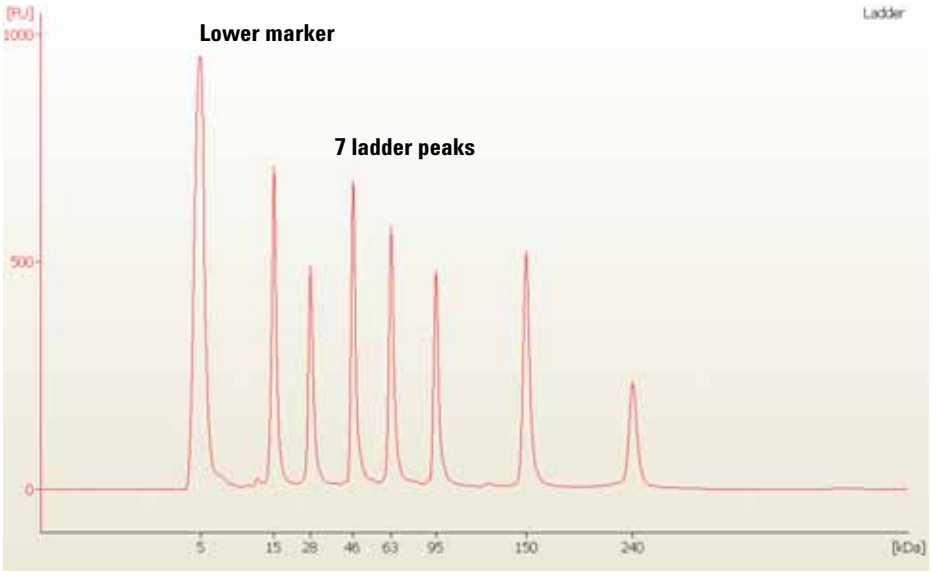


Figure 1 High Sensitivity Protein 250 ladder

Major features of a successful ladder run are:

- 7 ladder peaks and all peaks are well resolved
- 1 lower marker peak, usually higher than the ladder peaks

- Flat baseline
- Readings at 50 units higher than baseline readings

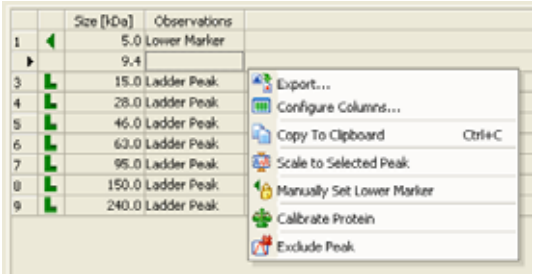
If the electropherogram of the ladder run does not resemble the one shown above check the handling of the labeling reaction and the handling of the on-chip analysis. For further and latest hints check the frequently asked questions section on Agilent's Lab-on-a chip website. For general assistance refer to the *2100 Expert Maintenance and Troubleshooting Guide* within the online Help of the 2100 Expert software.

NOTE

The High Sensitivity Protein 250 assay uses the ladder for relative quantification. It works without upper marker as internal standard. Alignment of Ladder and Samples is based on the Lower marker only.

In case wrong peaks are identified as ladder peaks, exclude them by doing the following:

1. Move the cursor over the peak in the peak table and click the right mouse button.
2. Select *Exclude Peak* from ladder to make the change come into effect.



High Sensitivity Protein 250 Sample Well Results

To review the results of a specific sample, select the sample name in the tree view and highlight the Results sub-tab. The electropherogram of the sample run should resemble the one shown below for the High Sensitivity Protein 250 assay.

Major features of a successful protein sample run are:

- A lower marker peak is visible.
- Baseline prior to the lower marker peak is flat.
- All sample peaks are migrating later than the lower marker peak and are resolved from it (depending on sample).
- The lower marker migrates between 16 and 24s (analysis turned off).
- The baseline reading is between 5 and 80 FU (analysis turned off).

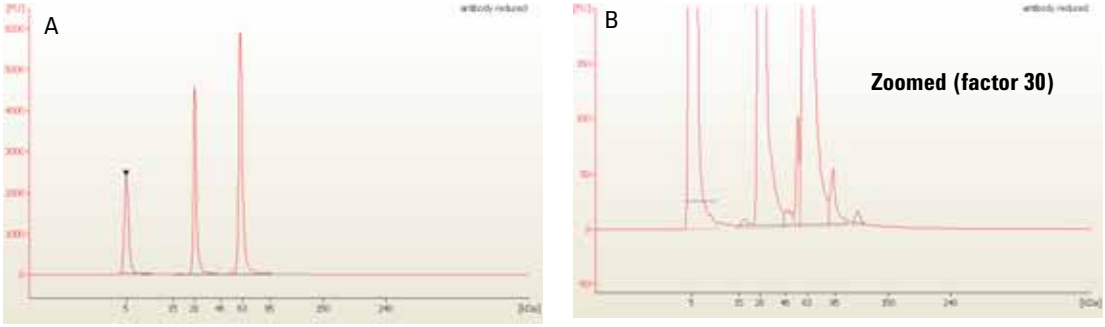


Figure 2 Sample lane, reduced antibody (A) full scale (B) Zoom-In

NOTE The Lower marker peak represents lower marker (a fluorescent dye) in the sample buffer plus excess of labeling dye. Therefore the overall Lower marker peak height is higher in case of low protein input to the labeling reaction compared to reactions with high protein input.

Insufficient dilution of labeling reactions will yield a saturation of the 2100 bioanalyzer fluorescence detector and subsequently an error message (optical signal too high). The electropherogram will show rectangular peak shapes for the lower marker or for dominant sample peaks. Sizing of all peaks will be affected since peak finding for the lower marker is biased. Quantitation of distorted sample peaks is affected due to the peak shape. Tailing of the lower marker peak beyond 10 kDa may disturb integration of smaller protein peaks.

Dilution of labeling reaction by 1:200 is recommended (page 24). For alternative workflows it must be estimated or determined experimentally.

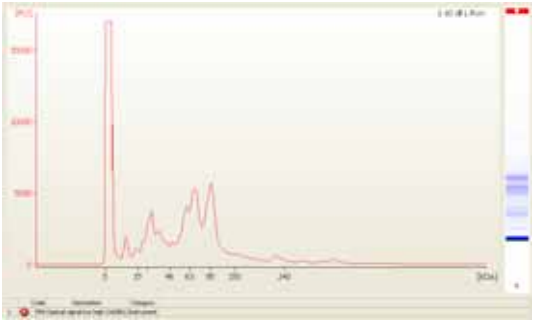


Figure 3 Detector saturation, choose higher dilution factor

NOTE In case the Lower marker peak disturbs the analysis excess of labeling dye from the reaction mixture may be depleted. Gel filtration can be employed to yield smaller lower marker peaks and to avoid the saturation of the fluorescence detector. This approach generally allows application of lower dilution factors and may increase sensitivity.

Quantitative Evaluation of Sample Results

Relative quantitation

For the High Sensitivity Protein 250 assay quantitation is done with the help of the ladder time corrected area. The sum for the identified 7 ladder peaks is compared with the sample peak. The area under the “lower” marker is not taken into consideration. Because the concentration of the Ladder is known (4167 pg/µl; initial Ladder concentration is 10^6 pg/µl and undergoes 5/6 dilution during labeling and 1:200 in dilution with water), the concentration for each sample peak can be calculated by the rule of three.

The result table gives the relative concentration. By default it refers to the concentration of the sample solution after dilutions. Please multiply the result with the dilution factors (standard protocol: 5/6 *labeling reaction* plus 1:200 *water dilution* equals 1:240 in total) to receive the concentration of you initial sample solution.

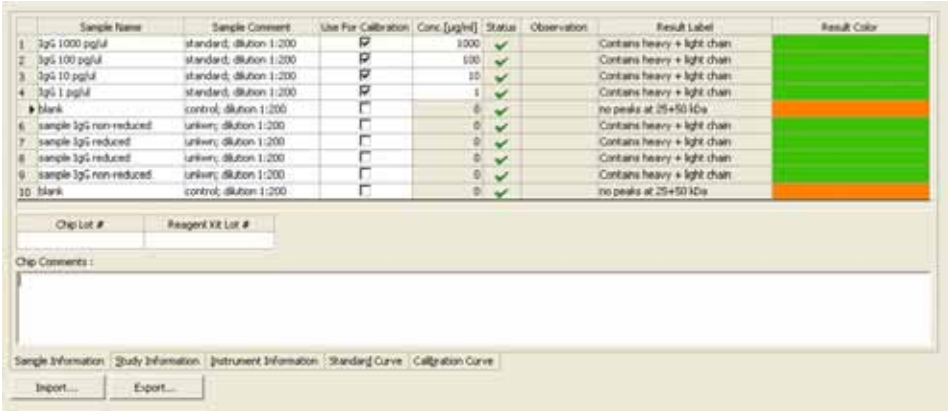
Besides a relative quantitation based on the ladder, an absolute quantitation is available using external standard proteins (see “Absolute quantitation” on page 42).

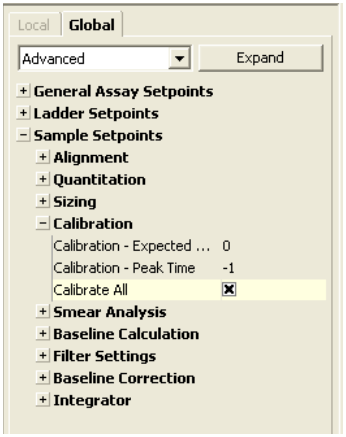
Internal standards spiked into the sample solution prior to the labeling reaction enable an alternative quantitation strategy. Introduction of a known peak concentration and employing the “% of Total” result from the analysis allows concentration calculation. The approach using an internal standard allows to control for matrix-related labeling effects.

NOTE Correct assignment of the ladder peaks is required for correct relative quantitation. Please check this for each run.

Absolute quantitation

If absolute quantitation is required with a standard protein, mark the check box **Use For Calibration** and enter standard concentration.

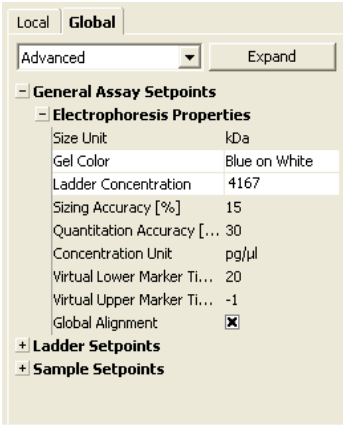




The highest peak in the calibration samples will be used for a linear calibration curve, which will be available on a new tab in the Chip Summary. The linear regression from time corrected areas of the calibration peaks are applied to the time corrected area of any sample peak to calculate the peak concentration.

Peaks from samples showing the same size as the calibration peak or manually assigned peaks will be quantified using the calibration curve. By default only one peak per lane is recognized as calibrated protein. In case all sample peaks should be evaluated with the calibration curve select the function “calibrate all” under advanced global setpoints from the setpoint explorer.

Adjustment of the ladder concentration



The dilution step of labeling mixes of sample and ladder is variable by the nature of the assay. The proposed dilution is 1:200 in water. Preset ladder concentration in the 2100 Expert Software is thus 4167 pg/µl. If different dilution ratios are used the total ladder peak concentration may be adjusted accordingly under advanced setpoints.

Another helpful customization is to adjust the ladder concentration setpoint to 10^6 pg/µl, i.e. the initial ladder concentration prior to the labeling reaction. Manual correction for the dilution factors (standard protocol: 5/6 *labeling reaction* plus 1:200 *water dilution* equals 1:240 in total) thus can be omitted. The concentrations given by the 2100 Expert Software will now refer to the initial sample concentration prior to the labeling reaction as long as samples are treated exactly like the ladder.

The Ladder Concentration setpoint may be considered as an adjustable factor for calibration purpose as well. In comparison to a well characterized master sample the ladder concentration value can be adjusted to let the assay yield the assumed concentration of this master sample. This adjusted value can be re-used in subsequent runs if the identical labeled ladder preparation is applied in the ladder well. This procedure allows to recycle a calibration result over several individual chips. Consider to generate a larger amount of labeled ladder in one reaction, e.g. 15 µl, to yield a sufficient number of identical aliquots.

Compatibility List for the Labeling Reaction

The following table lists protein sample buffers and buffer components which are known to have low impact on the labeling reaction. Others have a medium or strong negative influence. For an updated list please refer to the web-site www.agilent.com/chem/labonachip.

Please refer to the preparation of recommended buffers, see "Buffer Systems Suitable for the Labeling Reaction" on page 23.

Table 5 Compatibility List for the Labeling Reaction

Low Impact: 50% - 150% signal compared to 1xSLB*
30 mM Tris-HCl, pH 8.5 (Standard Labeling Buffer, 1xSLB *).
100 mM Sodiumbicarbonate* pH 9.0
1xSLB, 7 M Urea, 2 M Thiourea (Urea/Thiourea Buffer*)
1xSLB with 0.1 mM DTT
1xSLB with 1.0 mM DTT
1xSLB with 20 mM EDTA
1xSLB, 0.04% Sodiumazide
1xSLB, 1% CHAPS
1xSLB, 1% Triton
1xSLB, 1% Tween
100 mM Tris-HCl, pH 8.5
1xSLB + PBS (30 mM Tris-HCl, 26 mM NaH_2PO_4, 41 mM Na_2HPO_4, 79 mM NaCl) pH 8.5
50 mM HEPES, pH 8.0
50 mM CHES, pH 8.5

Table 5 Compatibility List for the Labeling Reaction (continued)

30 mM Tris-HCl, 1.25 M NaCl, pH 9.0
30 mM Tris-HCl, 30% Glycerol, pH 8.3
30 mM Tris-HCl, 0.9 M KCl, pH 9
30 mM Tris-HCl, 50 mM $MgCl_2$, pH 8.3
Medium Impact: 10% - 50% signal compared to 1xSLB*
1xSLB with 10 mM DTT
50 mM Tris-HCl, 100 mM NaCl, 20 mM Glutathion, pH 8.5
1xSLB, 1% SDS, pH 8.7
30 mM Tris-HCl, pH 7.3
Strong Impact: < 10% signal compared to 1xSLB*
100 mM Glycine/NaOH, pH 9
50 mM Tris-HCl, 500 mM NaCl, 500 mM Imidazole, pH 9

* These are recommended buffers for the labeling reaction.

List of Known Effects from Dilution Buffers

The following table lists tested matrices for on-chip analysis of labeled proteins, i.e. the compatible buffers for dilution of the labeling reaction or the matrix after following an alternative workflow.

Table 6 Tested matrices

Dilution Buffer	Sizing Effect	Comment
Water*, de-ionized	0%	Reference
Low Impact, Signal Intensity > 50%		
10 mM Tris, 1 mM EDTA (TE)	< 3%	
10% DMSO, water	< 3%	
50 mM CHES, pH 8.5	< 3%	
4 M Urea, water	< 3%	
40% Acetonitrile, 0.1% formic acid	< 3%	
1% SDS in water	< 3%	
30% Glycerol in water	< 3%	
Medium Impact, Signal Intensity 10% - 50%		
1 mg/ml BSA**, PBS (26 mM NaH_2PO_4, 41 mM Na_2HPO_4, 79 mM NaCl) pH 7.4	< 3%	
1xSLB	< 3%	
1xSLB, 100 mM NaCl	< 3%	
1xSLB, 100 mM KCl	< 3%	

Table 6 Tested matrices (continued)

Dilution Buffer	Sizing Effect	Comment
1xSLB, 100 mM DTT	< 3%	
100 mM Sodiumbicarbonate pH 9	< 3%	
PBS	< 3%	
PBS, 250 mM Imidazole	< 3%	
50 mM Sodium-Acetate, pH 5	< 10%	LM migration affected
1xSLB, 20 mM EDTA	< 10%	
PBS, 0.1% Tween-20	< 10%	
50 mM HEPES pH 7.5	< 10%	LM migration affected
50 mM MES, pH 6	< 10%	LM migration affected
50 mM Sodium-Phosphate, pH 7.5	> 10%	LM migration affected
50 mM MOPS, pH 7	> 10%	Migration time and Peak shape affected
Strong Impact, Signal Intensity < 10%		
50 mM Tris-HCl, 100 mM NaCl, 20 mM Glutathion, pH 8	< 3%	High ionic strength affects sample injection
30 mM Tris-HCl pH 8.5, 50 mM $MgCl_2$	< 3%	High ionic strength affects sample injection

* This is the recommended solution for the dilution.

** Non-labeled proteins will not be detected in the assay.

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In This Book

you find the procedures to analyze Protein samples with the Agilent High Sensitivity Protein 250 Kit and the Agilent 2100 bioanalyzer.

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- On-Chip Analysis of Labeled Proteins
- Agilent High Sensitivity Protein 250 Results
- Compatibility List for the Labeling Reaction
- List of Known Effects from Dilution Buffers

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